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EXPOSURE ASSESSMENT TO PATULIN FROM THE CONSUMPTION OF APPLE-BASED PRODUCTS

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Patulin is a mycotoxin produced by species of *Penicillium, Aspergillus* and *Byssochylamys*. Several Scientific Committees classify patulin as mutagenic, embriotoxic and immunotoxic. It has been found as a natural contaminant of processed apple products and its presence may be indicative of the quality of fruit used in production. In this work a method for the analysis of patulin is described, this methodology is based on a simple liquid-liquid extraction with acetonitrile; patulin is analyzed using liquid chromatography with UV detection. Patulin identity was confirmed by GC-MS after its reaction with N-methyl-N-(trimethylsilyl) trifluoroacetamide. Fifty-three apple-containing products were analyzed with the method described in this work. Patulin was detected in 14 of the samples in a range of 1.5-50.9 μg L^{-1}; six of them were above the maximum permitted level by the European

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Union. Based on these results and juice consumption by the Spanish adult population, patulin estimated intake was 0.42 ng kg^{-1} body weight per day.

Keywords: apple products, estimated daily intake, patulin

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Introduction

Patulin is a mycotoxin produced by certain species of *Penicillium*, *Aspergillus*, and *Byssochylamys*; these fungi generally grow in the post-harvest period of apples and pears (Piemontese 2005). The optimal conditions for mycotoxin production by *P. expansum* are: pH=6 and 25ºC in pears and 17ºC in apples. However, patulin may be also formed in a temperature range of 0-25ºC; mycotoxin production is inhibited when the fungi are exposed to an atmosphere containing 3% CO_2 and 2% O_2 at 25ºC (Doores 1983; Paster 1995; Podgorska 1992).

The effects caused by this mycotoxin are based on several studies made during the past fifty years; these involve acute, chronic and cellular level effects. Patulin may be neurotoxic, immunotoxic, immunosuppressive, genotoxic, teratogenic and carcinogenic. The Ministry of Agriculture, Fisheries and Food from the United Kingdom has classified patulin as mutagenic (MAFF 1993). A study performed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) concludes that this mycotoxin does not have either teratogenic or reproductive effects, but it shows embriotoxicity along with maternal toxicity (JECFA 2002). At relatively high doses, patulin has immunotoxic properties (WHO 1995). However, there is not adequate

evidence that patulin has a carcinogenic effect over experimental animals and this effect has not yet been tested in humans; therefore, the International Agency for Research on Cancer (IARC) classified patulin in group 3 or as "not carcinogenic to humans" (IARC 1986).

The natural presence of patulin in foodstuffs, mostly in apple-based products, has raised certain international attention. Nowadays, the *Codex Alimentarius* recommends residual patulin concentration to be less than 50 μg kg^{-1} in apple-based food destined to human consumption (Codex 2003); in some countries, e.g. Lithuania, Poland and Slovakia, the maximum content in baby food and food destined for infants was set at 20 μg kg^{-1} and 30 μg kg^{-1}, respectively (FAO 2004). The European Union (EU) established a maximum level of 50 μg kg^{-1} in fruit juices and fruit nectars; and 10.0 μg kg^{-1} in the case of food for infants, young children and other baby food (European Commission 2006).

Patulin, 4-hydroxy-4*H*-furo[3,2-*c*]pyran-2(6*H*)-one, is an unsaturated heterocyclic lactone. It is a colourless, crystalline compound with a melting point of 110°C and with a maximum UV absorption at 276 nm. Earlier methods for the determination of patulin in apple juice utilized thin layer chromatography (TLC), like the AOAC original official method (method number 974.18); this TLC method involved ethyl acetate extraction and clean up on a silica gel column (Scott 1974). LC coupled to UV detection has been extensively used given the polar nature of patulin, its characteristic UV absorption spectrum and the requirement to accurately determine levels in a very low concentration range (Sydenham et al. 1995). Nevertheless, interfering compounds, like 5-hydroxymethylfurfural (HMF), can simultaneously elute with patulin from a reverse

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phase column; therefore, an alternative approach based on the use of gas chromatography-mass spectrometry (GC-MS) has been used to confirm the presence of patulin in apple juice as its trimethylsilyl (TMS) derivative or as underivatized patulin using on column injection (Lezi et al. 1981, Llovera et al. 1999, Rupp et al. 2000, Roach et al. 2002, Tabata et al. 2004).

Patulin is soluble in water and polar organic solvents, it is not destroyed by heat, and it is stable in acid medium but destroyed by alkaline pH or fermentation. Several methods for extraction and clean up of samples, mainly fruit juices such as apple and pear juice, have been established in recent years. In the AOAC official method 995.10, patulin is extracted with ethyl acetate and isolated by extraction with a sodium carbonate solution (Brause et al. 1996). Extraction with polar solvents such as ethyl acetate or acetone is widespread. The following clean up can be performed with column chromatography using silica gel 60, florisil or celite (Gokmen et al. 1999, Leggot et al. 2001). However, the described methodologies use considerable solvent volumes (c.a. 25 mL) and perform back to back extractions, therefore are time consuming.

Given the adverse effects that this mycotoxin may cause and the necessity to control its presence in foodstuffs; the objective of this work was to monitor patulin levels in apple-based products in order to determine the risk in its consumption by the Spanish population.

Materials and methods

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Chemicals

Patulin standard and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma (St. Louis, MO, USA). Patulin standard solution was prepared in ethyl acetate at 500 µg mL^{-1}; it was kept wrapped in aluminum foil at -20ºC. Patulin working solutions were prepared daily by evaporating to dryness a known volume of the standard solution and redisolving in mobile phase. LC grade acetonitrile was obtained from Merck (Darmstadt, Germany). Deionised water (0.125 µS) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Samples

A total of fifty-three apple-based products were purchased in different stores and supermarkets from Valencia, Spain; 17 apple juices and beverages, 18 apple purees and compotes, 6 apple-containing dairy products, 3 ciders, 6 apple-containing breakfast cereals and snacks and 3 apples.

Extraction procedure and clean up

Recovery of the extraction method was determined by sample fortification. When solid samples were to be analyzed, 3 g of sample were placed in a centrifuge tube and fortified one hour before extraction with a patulin working solution at 0.05 µg mL^{-1}; samples were extracted with 5 mL of acetonitrile and vortexed for 1 minute. Then, the mixture was centrifuged at 16ºC, 4000 rpm during 20 min. The supernatant was evaporated to dryness with N_2 at 40ºC. Once cooled, it was reconstituted in 500 µL of mobile phase.

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In the case of clear, cloudy apple juices and nectars, the same procedure was followed using 3 mL of sample.

Liquid Chromatography-UV (LC-UV)

A Merck Hitachi (Kyoto, Japan) L-7100 HPLC quaternary pump, a Rheodyne Model 7125 injector (20 µL loop) and an L-7400 UV-detector were used. Chromatographic conditions were: LC Phenomenex column Luna 5 µm C18 100 A (150 x 4.60 mm i.d.) with a mobile phase consisting of water:acetonitrile (95:5 v/v) at a flow rate of 0.5 mL min^{-1}. Detection of patulin was carried out using a wavelength of 276 nm.

Confirmation by gas chromatography/mass spectrometry (GC/MS)

Analysis was carried out using a Trace GC (ThermoElectron Corporation, San Jose, CA, USA) gas chromatograph with a ThermoFinnigan AS 2000 automatic injector and the Xcalibur 1.2 software. Mass spectrometric data was collected in full-scan and SIM modes. Full scan data was used for the preliminary selection of the best target m/z ions and qualifiers. The SIM mode was used to maximize sensitivity and selectivity.

The column utilized was a fused silica column DB-5ms (J&W Scientific, Folsom CA, USA) of 30 m x 0.25 mm i.d. column, d.f. = 0.25 µm. The carrier gas was helium at a constant flow rate of 1.5 mL min^{-1}. The oven temperature was programmed at 140 °C and was ramped at 5°C min^{-1} up to 190°C, after one min temperature further ramped at 30°C min^{-1} up to 260 °C, and then was held for 5 min. The GC-MS transfer line was held at 298°C and the ion source was fixed at 284ºC. The MS was used under EI

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(electronic impact) mode (-70 eV), the ions selected for patulin identification were as follows: *m/z* 136, 183 and 226.

Derivatization was made based on previous work by Melchert & Pabel (2004) with slight modifications; 100 µL of patulin working solution were derivatizated with 100 µL of MSTFA and kept 20 min at 60ºC in a heating block; the mixture was allowed to cool to room temperature for 15-20 min, then it was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 300 µL of acetonitrile. A 1 µL aliquot was then analyzed by GC-MS. All positive extracts were analyzed with this protocol.

Results and discussion

Extraction and clean up

Under the established chromatographic conditions, it is possible to analyze clear apple juice by direct injection, prior filtration, into the system given that at the retention time of patulin (15.82 min) there are no interfering peaks; however in order to have a lower detection limit and not cause accumulation of substrate in the column, the described extraction and clean up methodology should be utilized. Including acetonitrile in the mobile phase mixture at 5 % (v/v) resulted in good separation by faster elution.

Most methodologies for patulin use liquid-liquid extraction with ethyl acetate as extraction solvent, the quantity used is considerably higher than that of acetonitrile used with the proposed method. Other methodologies utilize clean up cartridges or enzymes to clarify the extract (Gokmen et al. 1999, Leggot et al. 2001), the proposed methodology allows avoiding the use of these elements either by centrifugation or

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proper chromatography conditions. In Figure 1b, the chromatogram of a naturally contaminated apple juice sample is shown; patulin concentration in this sample is 1.5 µg L^{-1}. No interfering peaks can be observed at the time patulin is retained.

[Insert Figure 1 about here]

It was decided to perform a confirmatory procedure in case of finding naturally contaminated samples, although LC methods for the determination of patulin have mostly been preferred, a number of GC methods have been developed (Tarter et al. 1991, Ehman & Gaucher 1997, Roach et al. 2000, Llovera et al. 2005) since mycotoxins from the group of trichothecenes and patulin are readily derivatized to their TMS-derivatives and can be separated by 30m fused-silica capillary columns like the one used in this study. This separation can be appreciated in Figure 1c.

Method performance

Mean recovery of fortified apple juice and puree samples (n=5) at a level of 10 ng mL^{-1} of patulin was 90.6 % with a relative standard deviation of 6.2 %, recovery remains almost unchanged when working at levels of 5 and 15 ng mL^{-1}; in these cases recovery values were 87.8 ± 5.4 % and 89.1 ± 6.1 %, respectively. Intra-day (n=5) and inter-day (5 different days) variation values at a fortification level of 10 ng mL^{-1} were 4.4 and 6.3 %, respectively. These values do not exceed 15 %, which is the maximum variation for certification exercises for several mycotoxins (Hald et al. 1993). To estimate the limit of detection (LOD), apple juices and puree samples spiked at concentration levels of 10.0, 3.0, and 1.0 ng mL^{-1} were extracted and analyzed using the proposed procedure, the LOD and limit of quantification (LOQ) values were calculated by applying the 3σ

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criterion (Wenrich et al. 2000). The limits of detection and quantification for the optimized method were 0.1 and 0.3 ng mL^{-1}, respectively.

Results indicate good recovery and reproducibility of the method proposed in this work. This method offers several advantages, e.g. the quantity of solvents used is lessened and it does not require a large sample amount. Therefore, it is a good alternative that allows performing analysis with good precision and accuracy.

Incidence of patulin in apple-based products

Fifty-three apple-based products were analyzed. As it can be seen in Table 1, patulin was detected in 26.4 % of the samples, mainly in apple juices and purees; six of these samples had a patulin concentration over the maximum recommended limit in the EU.

The high incidence of patulin found in this study may be indicative of the poor quality of fruit used in production, given that patulin producing fungi may grow in the post-harvest period of pears and apples even at low temperatures. However, growth of the blue mould (a distinctive characteristic of *P. expansum*) and subsequent production of patulin is normally associated in those cases where the surface tissue of fruit has been damaged, although the presence of patulin in otherwise visibly healthy fruit can not be discounted. Fruit cleansing procedures and removal of damaged tissue, immediately before pressing, do not necessarily eliminate all patulin present since patulin migration to healthy tissue may be up to 1 cm (Taniwaki et al. 1992). The problems that carry patulin intake and all mycotoxins in general may get worst if organic agriculture becomes more popular in the future. A patulin concentration of 244-3993 μg L^{-1} has

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been reported in conventional apple juice (made from apples where pre and post-harvest synthetic fungicides have been used); meanwhile in apple-based juices of organic production, patulin levels up to 45000 µg L^{-1} have been found. Patulin has also been found in legumes, moist cereals, feeds, bread with levels ranging from 20-300 µg kg^{-1} and in beet from 12-3700 µg kg^{-1} (Lovejoy 1994, Trewavas 2001). Fermentation processes destroy patulin, this is probably why this mycotoxin is not often found in products like cider, unless apple juice was used after fermentation with the purpose of homogenize alcohol concentration in the batch.

[Insert Table 1 about here]

The results on patulin contamination found in this study are similar to those reported in other countries; in Italy, a survey on the occurrence of patulin was conducted on commercial puree apple juices and mixed apple juices. Patulin could be quantified in 34.8 % of the samples ranging from 1.58 to 55.41 µg kg^{-1} (Spadaro et al. 2007). Burda (1992) found patulin in 23 % of the fruit product samples from the United Kingdom and 22 % of these samples contained patulin levels between 51 and 1130 µg L^{-1}. Among 100 apple juice samples surveyed for incidence of patulin in Spain, 82 samples were contaminated with a mean of 13.8 µg L^{-1} and seven of these samples had patulin levels above 50 µg L^{-1} (Prieta et al. 1994). In Iran, 33 % of the apple juice samples had patulin levels higher than 50 µg L^{-1} with a maximum level of 285 µg L^{-1} and 56 % of the apple juice concentrates with a patulin level higher than 50 µg L^{-1} and a maximum level of µg L^{-1} (Majid Cheraghali et al. 2005).

Estimated daily intake of patulin

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Based on the results from this study on patulin contamination of apple juice and data on juice consumption by the Spanish population, patulin daily intake was estimated to be 0.42 ng kg^{-1} body weight per day (29 ng per person). This value was calculated considering the body weight for the Spanish population (68 kg) used in the "Assessment of dietary intake of Patulin by the population of EU Member States" (JECFA 2002); and it is in agreement with the data presented in the forementioned report (12.06-54.27 ng per person). Patulin intake does not represent a risk for the adult consumer given that the JECFA changed the provisional tolerable weekly intake (PTWI) of 7 μg kg^{-1} body weight per week to a provisional maximum tolerable daily intake (PMTDI) of 0.4 μg kg^{-1} body weight per day, based on a non observable effect level (NOEL) of 43 μg kg^{-1} body weight per day and a safety factor of 100 (WHO 1995). Table 2 presents patulin intake values based on patulin contamination data from this work and apple-based product consumption data found in literature.

Based on the data exposed in this paper; by comparing patulin intake reported in Table 2 against the PMTDI, it could be stated that patulin present in apple based products contributes from 0.1-21.6 % to the level considered at risk for human health caused by patulin exposure in Spain alone; therefore it does not represent high risk for the consumer of these products; however there is a higher risk for those groups more vulnerable, like babies and children, where the consume of these products is superior regarding to their body weight.

[Insert Table 2 about here]

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These intake data is for orientation purposes only, given that in Spain there is not official specific data on apple juice consumption. Despite of this, it is important to take notice on the products that exceeded the maximum recommended level for patulin, especially in products destined to infants and babies; therefore, measures to reduce patulin level in this kind of products must be taken.

In conclusion, results indicate good recovery and reproducibility of the method proposed in this work. It is a good alternative that allows performing analysis with good precision and accuracy given that with this methodology; the volume of solvent used for extraction is lessened being friendlier with the environment and cost effective. Patulin was found in 26.4 % of the samples analyzed, however the estimated patulin daily intake does not represent a serious risk for the adult consumer since it is far below the PMTDI; but attention must be paid in groups with higher risk of exposure like infants.

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Figure caption

Figure 1. a) Patulin working solution at 1 µg/mL, b) Naturally contaminated apple juice sample with a patulin content of 1.5 µg/L, c) Gas chromatogram of TMS-patulin from an apple juice sample.

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Table 1. Incidence of patulin in apple-based products.

Product	Incidence	Range ($\mu g\ L^{-1}$ or $\mu g\ kg^{-1}$)
Apple juice and apple-containing beverages	5/17	1.5-50.9
Apple compote and purees	6/18	7.7-28.4
Apple-containing dairy products	3/6	4.2-15.2
Cider	0/3	nd
Apples	0/3	nd
Apple-containing breakfast cereals and snacks	0/6	nd
Total	14/53	1.5-50.9

nd: not detected, under the LOQ

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Table 2. Estimated patulin intake from apple-based products

Product	Individual daily consume[(a)] (mL)	Body weight (kg)	Patulin content[(b)] (µg L^{-1} or µg kg^{-1})	Patulin daily intake (ng kg^{-1} body weight)
Fruit juice	6	68	4.8	0.4
Apple juice	50	20	4.8	12.0
Apple juice	61	60	4.8	4.9
Apple juice	216	12	4.8	86.4
Apple juice	200	64	4.8	15.0

[(a)] Apple based products consume by some Spanish population sectors reported at Reports on tasks for scientific cooperation 2002, Gimeno et al. 2003, Legarda & Burdaspal 2005.

[(b)] Average patulin content from the apple juice and apple-containing beverages studied in this work.

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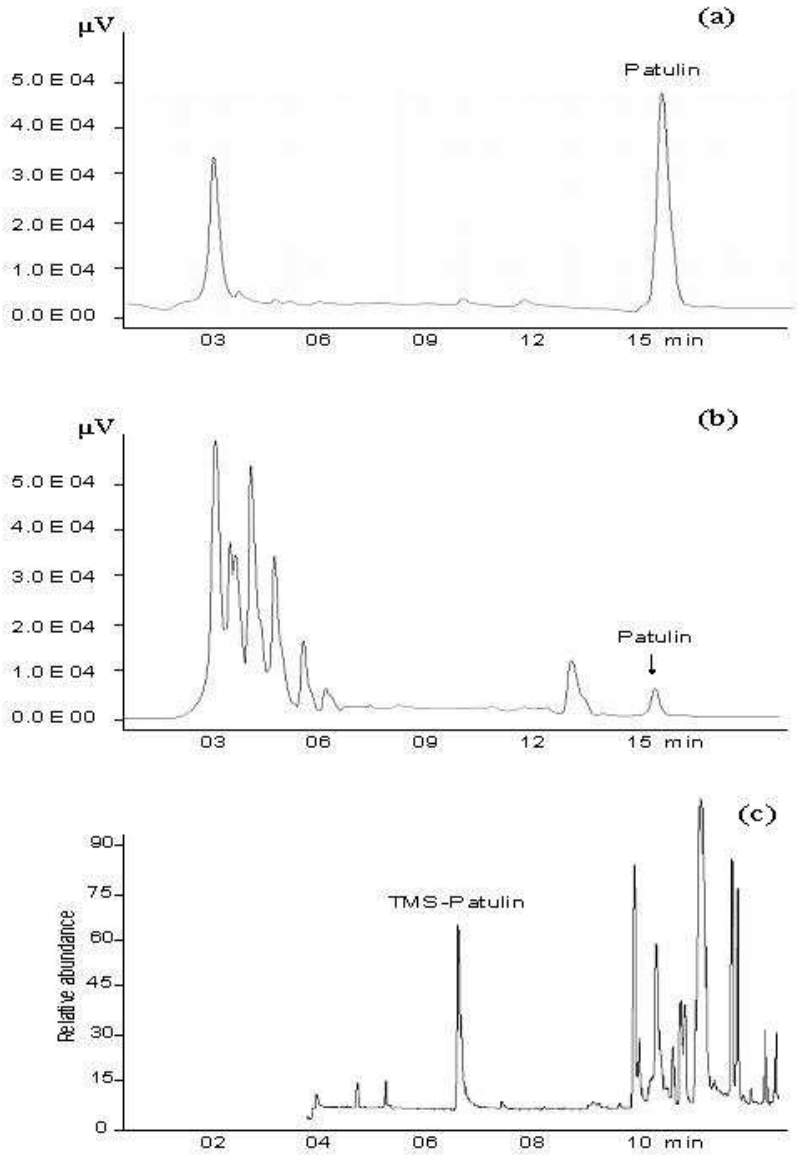

Figure 1. a) Patulin working solution at 1 µg/mL, b) Naturally contaminated apple juice sample with a patulin content of 1.5 µg/L, c) Gas chromatogram of TMS-patulin from an apple juice sample.
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